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Report

Visual Targeting of Motor Actions in Climbing *Drosophila*

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Summary

Drosophila melanogaster **flies cross surmountable gaps in their walkway of widths exceeding their body length with an astounding maneuver but avoid attempts at insurmountable gaps by visual width estimation [1]. Different mutant lines affect specific aspects of this maneuver, indicating a high complexity and modularity of the underlying motor control [1]. Here we report on two mutants, *ocelliless*^{1} and *tay bridge*^{1} [2], that, although making a correct decision to climb, fail dramatically in aiming at the right direction. Both mutants show structural defects in the protocerebral bridge, a central complex neuropil formed like a handlebar spanning the brain hemispheres. The bridge has been implicated in step-length control in walking flies [3] and celestial E-vector orientation in locusts [4]. In rescue experiments using *tay bridge*^{1} flies, the integrity of the bridge was reestablished, concomitantly leading to a significant improvement of their orientation at the gap. Although producing directional scatter, their attempts were clearly aimed at the landing site. However, this partial rescue was lost in these flies at a reduced-visibility landing site. We therefore conclude that the protocerebral bridge is an essential part of a visual targeting network that transmits directional clues to the motor output via a known projection system [5].**

Results and Discussion

Climbing of Wild-Type and Protocerebral-Bridge-Defective Flies

Although wild-type flies have an average body length of only 2.5 mm, they are able to surmount gaps of up to 4.3 mm with a special climbing behavior [1]. To successfully cross challenging widths, flies have to avoid deviations from the optimum direction because any angular deviation will shorten their effective reach when leaning into the gap and trying to reach the other side with their front legs. After contacting the opposite side with their front legs, the middle legs are released and also reach for the landing side. Finally, the hind legs release their grip and the flies walk up the opposite vertical wall and over the edge to continue onto the horizontal surface. To assess the angular precision, we measured the deviation in the x-y plane as the angle between the optimal climbing direction and the longitudinal axis of the fly (Figures 1A and Aa; frames taken from high-speed motion pictures recorded at 200 frames/s; see Movie S1). The orientation was determined at the time of the last front-leg stroke before either making contact to the other side or giving up. These distinct leg-over-head search strokes of the front legs are a unique sign of a climbing attempt because they do not occur during normal walking [1]. Wild-type Berlin (WTB) males showed a median scatter of just 6.2° when monitoring 58 attempts from ten flies crossing a 3.5 mm gap (Figure 1D). A more detailed statistical analysis is given in Figure 1G, which shows the absolute angular deviation from the optimal climbing direction in terms of median, 25%, and 75% quartiles (the whiskers denote the entire range of angles found).

The low scatter suggested that flies are actively aligning their body axis in relation to the gap. To determine whether the central complex, and specifically the protocerebral bridge, plays a role in this orientation behavior, we tested mutant flies with defective protocerebral bridges. The bridge is a neuropil shaped like a bicycle's handlebar within the central complex, which interconnects the protocerebral hemispheres of the insect brain (Figure 1D). It consists of eight glomeruli per hemisphere and is interconnected with the other three neuropilar regions of the central complex called fan-shaped body, ellipsoid body, and the paired noduli. The connectivity is established by columnar projection systems that come in sets of homologous neurons in multiples of eight [5]. Most of these systems carry output from the bridge; few provide input. Tangential elements interconnect the glomeruli of the bridge intrinsically.

By electrophysiological means a map-like representation of the head orientation relative to the E-vector of polarized light has been found in the protocerebral bridges of locusts [4, 6]. We have previously shown that *Drosophila* mutants with lesions in the protocerebral bridge (*tay bridge*^{1}) are defective in controlling their step size [3]. Another mutant strain with a severe defect of the protocerebral bridge (*ocelliless*^{1}*; oc*^{1}) was originally described as lacking the three simple eyes on top of the head and later identified as an allele of *orthodenticle* (*otd* [7–9]). With the exception of the two outermost glomeruli (no. 8), the bridge is missing in *oc*^{1} flies, and only occasionally *oc*^{1} flies show additional small fragments of bridge material. *oc*^{1} flies readily climbed 3.5 mm wide gaps but with a significantly lower success rate than wild-type flies (21% ± 7% versus 56% ± 6%; $p < 0.001$, two-tailed t test). The initiation rate of *oc*^{1} flies at this surmountable gap width is not the cause of their low success (78% ± 7% versus 78% ± 4%; not significantly different, two-tailed t test). Analyzing their orientation behavior revealed a highly significantly broader scatter of their climbing directions (Figure 1G; $p < 10^{-6}$, U test against WTB, Bonferroni corrected for multiple comparisons; statistical data are given in Table S1 available online). As shown in Figures 1B and 1E, many attempts are directed into the void, which never occurred in wild-type flies (Movie S2). Analyzing 134 attempts from 19 flies, we found a median angular deviation of 15.2° (Figures 1E, 1G, and 1H). To exclude the possibility that the missing ocelli are responsible for the increased angular scatter, we also tested wild-type flies that had their ocelli painted with light-tight black paint. Because the climbing precision of these flies was indistinguishable from that of unaltered wild-type flies (median 6.2°, Figure 1G; U test, corrected for multiple testing; Table S1), we conclude that the ocelli do not play a role in the orientation behavior.

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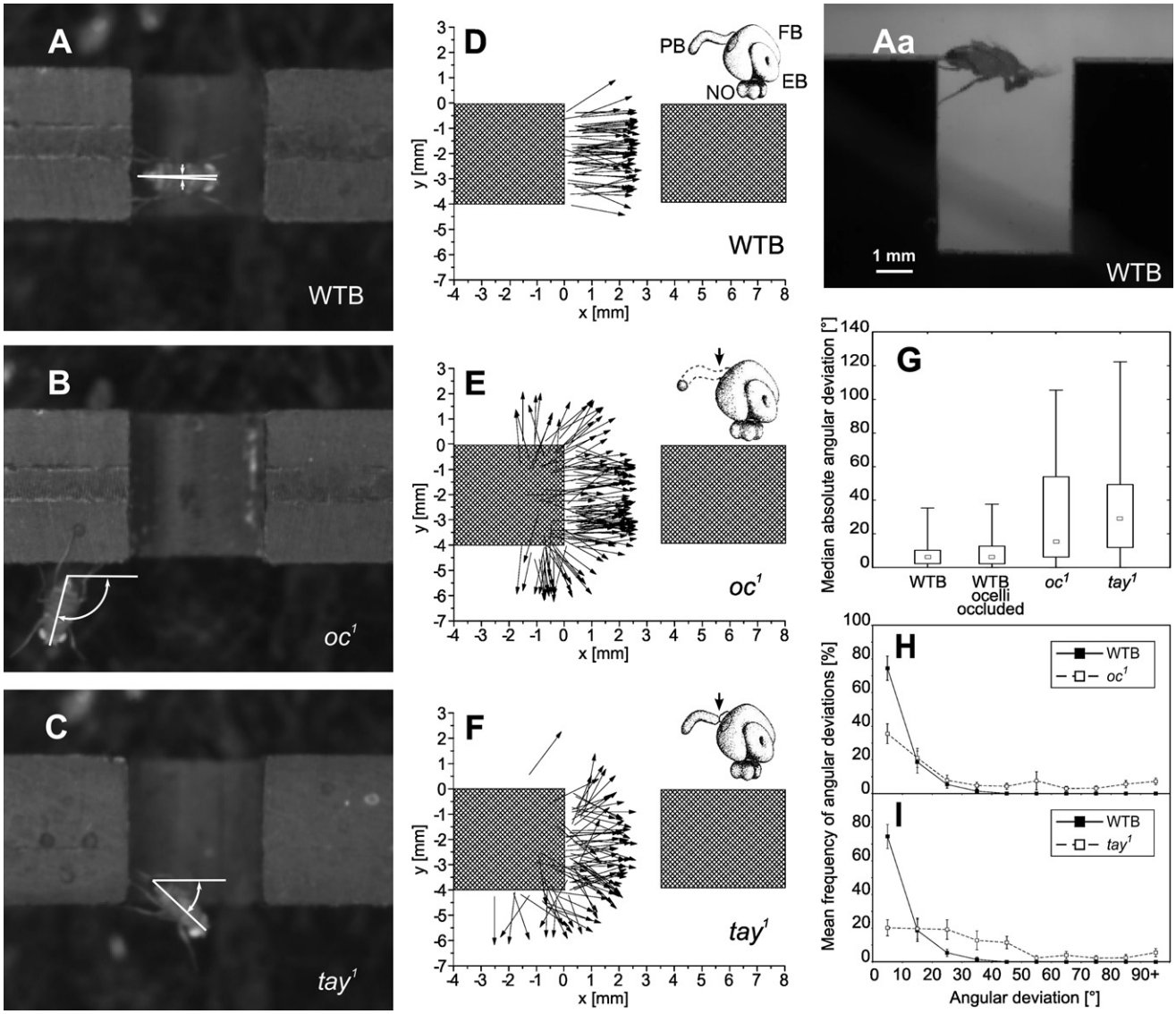

Figure 1. Wild-Type and Mutant Flies at the Standard Gap

(A–C) Examples of climbing attempts of a wild-type Berlin (WTB), an *ocelliless*^{1} (*oc*^{1}), and a *tay bridge*^{1} (*tay*^{1}) male fly. Angular deviations from the optimal direction are indicated. Gap width is 3.5 mm.

(D–F) Scatter of the orientation and position of the longitudinal body axis taken at the last stroke of a front leg before giving up or succeeding in contacting the opposite side. WTB: 58 events (n) from 10 flies (N); *oc*^{1}: n = 134, N = 19; *tay*^{1}: n = 82, N = 18. Sketches indicate the phenotype of the central complex (PB, protocerebral bridge; FB, fan-shaped body; EB, ellipsoid body; NO, noduli).

(G) Median absolute angular deviations from the perfect crossing direction (small rectangles). Boxes indicate 25% and 75% quartiles; whiskers denote the entire range of angular deviations. Data were taken from (D–F) and WTB with occluded ocelli added: n = 42, N = 5. The mutant data are highly significantly different from both WTB data sets. The full statistical account is given in Table S1.

(H and I) Mean frequency of angular deviations for *oc*^{1} and WTB (H) as well as *tay*^{1} and WTB (I) in 10° bins. Error bars denote standard error of the mean (SEM) values.

To test whether defects of the protocerebral bridge are indeed leading to defects in orientation, we analyzed the climbing behavior of a genetically independent protocerebral-bridge-defective mutant line called *tay bridge*^{1} (*tay*^{1}). *tay*^{1} flies show a medial constriction of the bridge and have been originally isolated in a screen for walking-impaired flies [2]. The analysis of 82 climbing attempts from 18 different male *tay*^{1} flies revealed again a conspicuously broader angular scatter (median 28.9°, Figures 1C, 1F, and 1I) statistically indistinguishable *from oc*^{1} (p = 0.322, U test, corrected for multiple testing; Figure 1G; Table S1). Also similar to *oc*^{1}, the climbing attempts of *tay*^{1} flies often occurred sideways into the void.

Rescue Experiments

To verify the causal relationship of the structural defects in the bridge with the observed orientation deficits, we tried rescuing the *oc*^{1} bridge by using a cDNA transgene of *otd*. However, inducing the UAS-*otd* transgene with all the GAL4 lines driving expression in the adult bridge resulted in lethality during development (data not shown). Therefore, we performed rescue

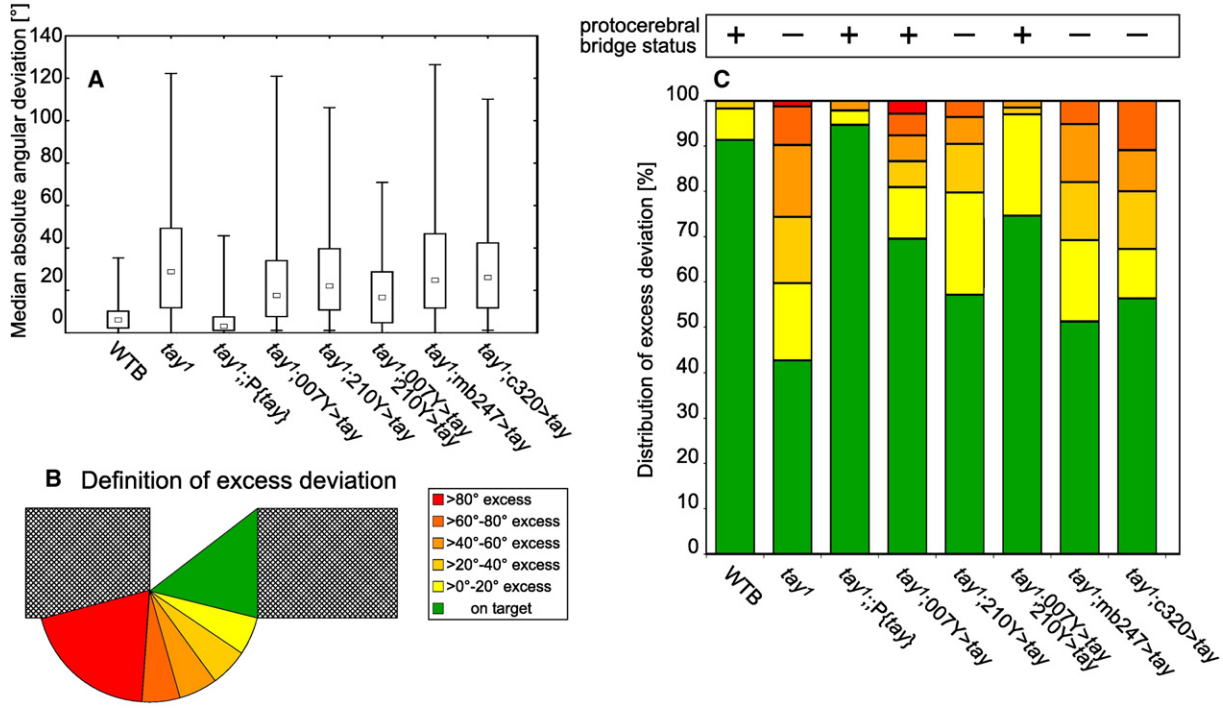

Figure 2. *tay*^{1} Rescue Flies at the Standard Gap

(A) Median absolute angular deviations of WTB, *tay*^{1} (data as in Figure 1), and various rescue lines. Only the genomic rescue *tay*^{1};;P{*tay*} restores all phenotypes (N = 8 flies, n = 94 events). *tay*^{1};UAS-*tay*;007Y-GAL4 (N = 32, n = 173), *tay*^{1};UAS-*tay*;210Y-GAL4 (N = 18, n = 84), *tay*^{1};UAS-*tay*;mb247-GAL4 (N = 7, n = 39), and *tay*^{1};UAS-*tay*;c320-GAL4 (N = 7, n = 55) do not rescue the angular deviation. Rescue flies that contain both drivers, 007Y-GAL4 and 210Y-GAL4 (N = 11, n = 67), show an intermediate phenotype between WTB and *tay*^{1}. Boxes indicate 25% and 75% quartiles; whiskers denote the entire range of data. For statistics, see Table S2.

(B) Excess deviation of events missing the opposite side is defined as the angle between the alignment of the fly that would just hit the other side and the actual body direction at the last leg-over-head stroke.

(C) Distribution of excess deviations in 20° bins from the data set in (A). Besides the genomic rescue, the driver line 007Y-GAL4 and the combination driver line 007Y-GAL4+210Y-GAL4 rescue excess deviation. In addition, these lines are the only ones with a restored protocerebral bridge, indicated by "+" for the wild-type or "-" for the *tay*^{1}-phenotype. The 210Y-GAL4 rescue, although it does not visibly restore the bridge, shows a somewhat intermediate behavioral phenotype. Statistical data are given in Table S3.

experiments with the *tay*^{1} mutant. A genomic rescue construct for *tay* (P{*tay*}) had completely rescued all known phenotypes of *tay*^{1}, including the structural defect of the protocerebral bridge [2]. It also rescued the angular deviation in the current study, providing additional evidence for the importance of the bridge in orientation behavior (median 3.1°, Figure 2A; not significantly different from WTB, Kruskal-Wallis test, corrected for multiple testing; full statistical account is given in Table S2).

To specifically address the functional role of the bridge, we then induced the UAS-*tay* construct with 007Y-GAL4, which had also been shown in the earlier study to rescue the structural defect of *tay*^{1}. 007Y-GAL4 expresses in the w, x, y, and z bundles of the central complex, which are formed by columnar projections between the bridge, the fan-shaped body, and the ventral bodies ([5]; Figure S1). These neurons, called the horizontal fiber system, have postsynapses in the bridge, mixed terminals in the fan-shaped body, and presynapses in the ventral bodies as judged by the Golgi gestalt [5]. Besides the expression in the horizontal fiber system, 007Y-GAL4 induces weak expression in the outer perimeter of the ellipsoid body, in a dorsal and a ventral-to-middle layer of the fan-shaped body, in the dorsal ends of the noduli, and in most parts of the mushroom bodies [2].

Comparative expression analysis of 007Y-GAL4 with a UAS-tau::GFP reporter construct revealed a significant reduction of the GFP expression in the glomeruli of the bridge in *tay*^{1} mutant flies. This reduction in staining intensity reflects a reduction in dendritic arborizations of the horizontal fiber system. This missing arborization most likely causes the medial constriction of the bridge as observed in autofluorescent micrographs of the *tay*^{1} mutant ([2]; Figure S2). Although we confirmed that UAS-*tay* expression via 007Y-GAL4 restored the integrity of the protocerebral bridge, as assessed by autofluorescent sections and the reconstitution of glomerular staining (Figure S1C), the behavioral defect in gap orientation was still present in these individuals. Indeed, the angular scatter of these rescue flies was not statistically different from mutant *tay*^{1} animals (median 17.7°, Figure 2A; p = 0.782, Kruskal-Wallis test, corrected for multiple testing; Table S2). Nevertheless, we noted that the rescue flies were more successful in their climbing attempts than the *tay*^{1} mutant flies. Although the scatter was still large, the attempts were more often directed to the opposite block. We therefore sorted the 173 attempts of the rescue flies into successful and unsuccessful events, whereby the latter were further grouped by the excess deviation by which the longitudinal body axis angled away from the opposite block (Figure 2B). Data were sorted into 20° bins for the graphical representation in Figure 2C. In terms of the excess deviation, the climbing behavior of the 007Y rescue flies was not significantly different from WTB and was highly

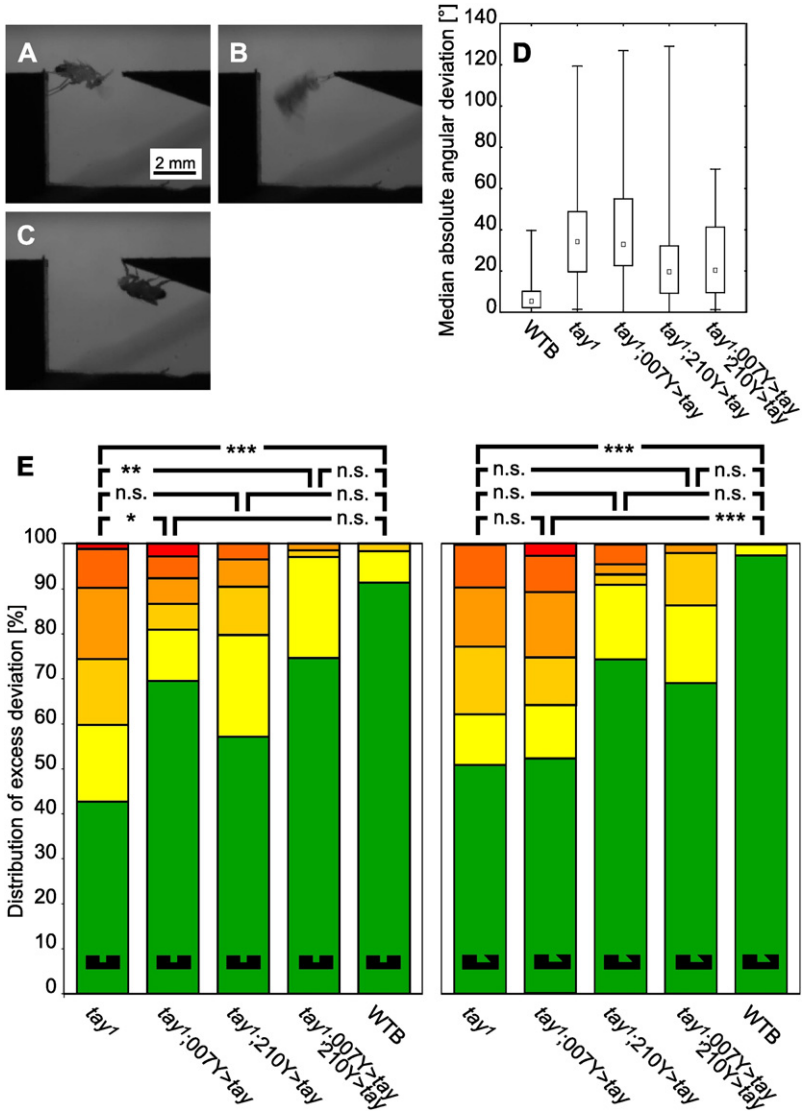

Figure 3. Climbing at the Reduced-Visibility Gap

(A–C) Three frames taken from a video sequence of a wild-type Berlin fly crossing the gap with reduced visibility of the contralateral side. (A) Last front leg stroke before touching the distal side. (B) Bridging the gap and release of the hind and middle legs. (C) Successful arrival at the distal side.

(D) Median absolute angular deviations from the ideal crossing direction at the reduced visibility gap (rectangles). Boxes indicate 25% and 75% quartiles; whiskers denote the entire range of data. WTB, n = 80 events, N = 12 flies; *tay*^{1}, n = 53, N = 11; rescue *tay*^{1};UAS-*tay*;007Y-GAL4, n = 75, N = 15; *tay*^{1};UAS-*tay*;210Y-GAL4, n = 90, N = 18, *tay*^{1};UAS-*tay*;007Y-GAL4+210Y-GAL4, n = 52, N = 10. Statistical data are given in Table S2.

(E) Comparison of the distribution of excess deviations at the standard (left) and reduced-visibility gap (right), taken from the data sets shown in 2C and 3D, respectively. Similar to the standard gap, the *tay*^{1};UAS-*tay*;210Y-GAL4 flies show a partial rescue of the excess deviation at the reduced-visibility gap. The full rescue observed with *tay*^{1};UAS-*tay*;007Y-GAL4+210Y-GAL4 at the standard gap reverts to a partial rescue at the reduced visibility gap, and the behavior of 007Y-GAL4 rescue flies, which is indistinguishable from WTB at the standard gap, does not differ from *tay*^{1} at the reduced visibility gap. n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001. The full set of p values is given in Table S3.

significantly smaller than that of *tay*^{1} (p = 0.367, p = 0.027, Kruskal-Wallis test, corrected for multiple testing; statistical analysis was performed on the excess deviation angles before binning; the full set of p values is given in Table S3).

To determine whether the expression of 007Y-GAL4 in the mushroom bodies plays a role in this effect, we expressed UAS-*tay* by the mushroom-body driver mb247-GAL4 [2]. As expected, expression of UAS-*tay* in the mushroom body did not rescue the protocerebral bridge, nor did it prevent the scatter (median 24.6°, Figure 2A; not significantly different from *tay*^{1}; Table S2) or the excess deviation (Figure 2C; not significantly different from *tay*^{1}; Table S3). This result and the concomitant rescue of the dendritic arborizations and of angular scatter by the 007Y driver suggest that the horizontal fiber system is required for this orientation task. Two other lines, 210Y-GAL4 [2] and c320-GAL4 [10], which both induce expression in the adult bridge, failed to rescue the structural *tay*^{1} defect when driving UAS-*tay* (Figure S2). Although c320-GAL4 addresses the horizontal fiber system in the adult bridge, no rescue of the primary scatter (median 26.0°, Figure 2A; not significantly different from *tay*^{1}; Table S2) or of the excess deviation was seen (Figure 2C; not significantly different from *tay*^{1}; Table S3). This negative result is most likely due to a delayed expression time point of c320 compared to 007Y.

In contrast, the driver line 210Y-GAL4 expresses in tangential neurons of the bridge [2] and therefore was not expected to rescue either the morphological or the behavioral phenotype. Interestingly, the primary scatter in 210Y-GAL4 rescue flies was not significantly different from *tay*^{1} flies (median 22.2°, Figure 2A; Table S2), but the excess deviation showed partial improvement (Figure 2C; not significantly different from *tay*^{1}, p = 0.062 against WTB, Table S3). These differences in the rescue abilities of the 007Y and 210Y driver lines could be due to their individual expression pattern within the protocerebral bridge or in other parts of the central brain where 210Y drives expression The latter interpretation might be supported by the rescue experiments using a recombinant 007Y+210Y-GAL4 driver line. This double-rescue experiment revealed that the effects of the 007Y and 210Y expression on the decrease of excess deviation are additive; these rescue flies were almost indistinguishable from WTB but dramatically different from *tay*^{1} (Figure 2C; n.s. and p = 0.004; Table S3).

Rescue Experiments at the Reduced-Visibility Paradigm

Because the 007Y rescue did restore the integrity of the protocerebral bridge but not the wild-type precision of the climbing direction, we tested whether the improvements of these flies might be attributable to a workaround solution. The protocerebral bridge could be involved in a visual targeting mechanism that enables the flies to target the comparatively broad front surface of the landing site. Such a mechanism is supported by previous observations that wild-type flies track the opposite front surface much more than the top surface [1]. Therefore, we made use of a top-side-only or diving-board-like

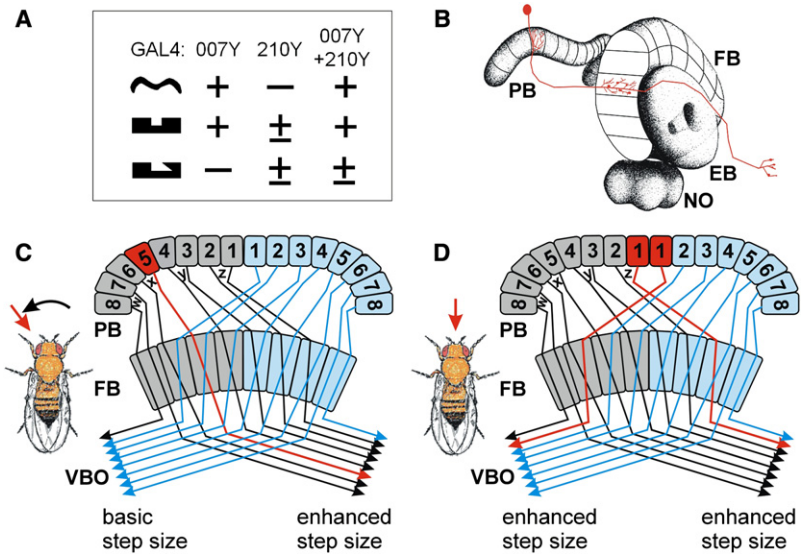

Figure 4. Anatomical and Functional Model of the Protocerebral Bridge

(A) Summary of the rescue experiments with regard to the structure of the protocerebral bridge and the excess deviation at the standard and low-visibility gap.

(B) Schematic of the central complex and a neuron of the horizontal fiber system (HFS; after [5]); PB, protocerebral bridge; FB, fan-shaped body; EB, ellipsoid body; NO, noduli. The HFS connects the two-times-eight glomeruli of the PB to the eight fans of the FB in a crossover scheme and exits to the ventral bodies (VBO). The HFS neurons have spiny arborizations in the PB, mixed terminals in layer 4 of the FB, and blebbed endings in the VBO.

(C and D) Functional model of the PB and HFS with the target sideways to the fly (C) and in front of the fly (D). The bridge is assumed to hold a representation of the target's azimuth position, which, by means of the HFS, influences step length contralaterally. As a consequence, the fly turns toward the object in (C) and speeds up in (D). w, x, y, and z denote w-, x-, y-, and z-fiber bundles, respectively, characteristic of the HFS [5]. Whereas 007Y-GAL4 induces expression in these fiber bundles, 210Y-GAL4 does not.

paradigm to investigate the effects of visual clues from the landing side (Figures 3A–3C). In this paradigm, wild-type flies have a lower climbing-initiation rate (this has also been noted earlier [1]) but no significant increase in the angular deviation (median 5.3°, compare to Figures 2A and 2C), nor in the excess deviation, of their attempts was observed (Figures 3D and 3E; Movie S3; statistical account is given in Tables S2 and S3). Expectedly, we also did not observe a worse phenotype in the *tay*^{1} mutant, which showed the same high angular deviation and excess deviation as in the standard paradigm with the solid opposite side (median 34.1°, Figures 3D and 3E; Tables S2 and S3). This finding is in accordance with the idea that the reduction of the visual cue by the low visibility of the opposite side is irrelevant to flies that cannot evaluate this cue because of their defect in the protocerebral bridge.

In contrast, 007Y-GAL4 rescue flies performed much worse in the low-visibility paradigm. Whereas the excess deviation was rescued when the solid standard block was used (Figure 2C), now these flies were indistinguishable from *tay*^{1} mutants (Figure 3E; not significantly different from *tay*^{1}; Table S3). This suggests that the increased performance with the standard gap is due to an improved visual targeting mediated by the restored protocerebral bridge. However, when the conspicuous visual cue of the landing site is removed, the restored bridge cannot provide this supportive function. This hypothesis is further supported by the results obtained with 210Y-GAL4, which does not restore the protocerebral bridge. In this case, the visibility of the opposite side is not of importance and consequently their performance remains the same in the diving-board paradigm as in the standard assay (Figure 3E, Table S3). Similarly, the combined driver line 210Y-GAL4+007Y-GAL4 does not show an improvement compared to 210Y-GAL4 alone, because the rescue of the protocerebral bridge by the 007Y component does not help when the visual cue is missing (Figure 3E, Table S3).

Conclusions

In recent years different laboratories provided evidence that multimodal sensory inputs into the central complex (e.g., E-vector orientation [4, 6, 11]; tactile input from the antennae [12]; visual orientation toward objects [2, 3, 13]) are used to establish a representation of the outside world. In this communication we could show that an intact protocerebral bridge is required to target the opposite side of the gap via acute visual information. This rescue is lost by removing the front surface of the landing side from the view of the fly. Therefore, this targeting mechanism depends on continuous visual input that is lost when only the diving board is presented during the climbing event. In contrast, the partial rescue provided by the 210Y driver line does not depend on such an online visual guidance because it persisted in the diving-board paradigm. This improvement could be due to a working memory that is not formed in the *tay*^{1} mutant (Figure 4A). Therefore, the 210Y rescue effect is additive to the 007Y rescue as seen in double-driver flies (Figure 4A). We therefore conclude that the protocerebral bridge holds a representation of the outside world (in this case of the vertical edges of the opposing surface) and the fly's orientation in it. The bridge most likely conveys preprocessed visual information through the ventral bodies to motor centers that finally steer the animal toward the indented direction.

In accordance with earlier findings on step-length control in bridge-defective flies [3, 14, 15] and described projection systems [5], we propose a structure-function model of the protocerebral bridge. The two-times-eight glomeruli of the bridge are connected by the horizontal fiber system to the one-times-eight fans of the fan-shaped body in a crossover scheme (Figures 4B and 4C). We suggest a model in which the visual target direction is represented in the bridge, the latero-lateral extent of which may code for the azimuth position of the chosen target. The sagittal glomeruli represent the frontal visual field, and the outermost glomeruli provide positional information from the rear of the fly. An asymmetrical representation corresponds to an object at the side of the fly and should enhance the step size on the contralateral body side by virtue of the output of the horizontal fiber system to the ventral bodies (Figure 4C), an accessory area of the central complex [5]. The body side ipsilateral to the target does not receive enhancement signals. It steps with a basic step length, and this asymmetry in step length causes turning toward the visual target. Notably, protocerebral-bridge-defective mutants show basic step length throughout and have problems turning [3, 14, 15]. When the fly is on target, the representation in the middle of the bridge may lead to an increase in step size on both sides

of the body and consequently an increase in walking speed, as has been found in WTB flies [16] (Figure 4D).

In a similar way, locusts and crickets could use the representation of the celestial E-vector in the protocerebral bridge to generate steering commands for their migration. The orientation of these insects with respect to the E-vector is preprocessed by neurons of the lower division of the central body (homologous to the ellipsoid body [4, 11]) and conveyed to the bridge [6]. Therefore, we can assume that the protocerebral bridge converts differences between world-centered and body-centered azimuthal coordinates into motor commands for orientation.

Experimental Procedures

Setup

A set of two orthogonal high-speed video cameras with 200 frames per second was used as described in [1]. The catwalk is 34 mm long, 10 mm high, and 4.0 mm wide and is interrupted by a gap in the middle of the longitudinal axis. The standard gap is rectangular, 3.5 mm wide, and 5.0 mm deep. The reduced-visibility gap is 5.0 mm deep, but whereas the gap is also 3.5 mm wide at the top it widens toward the bottom at the landing side, and the starting side has a vertical wall (Figures 3A–3C).

Flies

Flies were kept on standard *Drosophila* medium under a 14 hr/10 hr light-dark cycle at 25°C and measured at age 3–5 days. To prevent flying, wings were shortened to 1/3 of their length under cold anesthesia (4°C) in a stream of dry air. Flies were given at least 12 hr in food vials for recovery. Painting of the ocelli with Schmincke Aerocolor 28870 was done as described in [1]. WTB flies were taken from the culture at the Biocenter Wuerzburg; tay^1 has been created on a WTB background. The genomic rescue line P{*tay*} and the UAS-*tay* construct are described in [2]. oc^1 has originally been generated by Bedichek [7] by X-ray irradiation of an unknown genetic background. The driver lines 007Y-GAL4, 210Y-GAL4 [2, 17], and mb247-GAL4 [18] have a wild-type Canton-S genetic background [19]. c320-GAL4 is described in [20], and its expression pattern is shown in [10]. Male flies were used in all experiments, and all transgenes were in the heterozygous state.

Statistics

All data sets comprised groups that were not normally distributed (Shapiro-Wilk test). Pair-wise comparisons of Figure 1 were therefore performed with the Mann-Whitney U test, Bonferroni correction applied, and data are presented as medians, 25% and 75% boxes, and whiskers denoting the full range of the respective data set (Table S1). For all multiple-group comparisons, Kruskal-Wallis analyses of ranks were performed and tested post-hoc with Mann-Whitney U tests corrected for multiple comparisons [21]. The full sets of p and z' values are given in Tables S2 and S3. All analyses were done with Statistica version 7 (StatSoft).

Supplemental Information

Supplemental Information includes two figures, three tables, and three movies and can be found with this article online at doi:10.1016/j.cub.2010.02.055.

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